REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
13-09-2006		

4. TITLE AND SUBTITLE
Cloning, expression and purification of Brucella suis outer membrane proteins

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Xuan Z. Ding1, Apurba K. Bhattacharjee1, Ian T. Paulsen2, Mikeljon P. Nikolich1, Gary Myers2 and David L. Hoover1

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
1Department of Bacterial Diseases, Division of CD&I
Walter Reed Army Institute of Research, Washington, DC
503 Robert Grant Ave, Silver Spring, MD20910
2The Institute for Genomic Research, Rockville, MD 20850

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
This work was supported by the Department of Defense, JSTO/DTRA.

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Unlimited

DISTRIBUTION STATEMENT A
Approved for Public Release
Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Brucella, an aerobic, nonsporeforming, nonmotile Gram-negative coccobacillus, is a NIH/CDC category B bioterror threat agent that causes incapacitating human illness. Medical defense against the bioterror threat posed by Brucella would be strengthened by development of a human vaccine and improved diagnostic tests. Central to advancement of these goals is discovery of bacterial constituents that are immunogenic or antigenic for humans. Outer membrane proteins (OMPs) are particlularly attractive for this purpose. In this study, we cloned, expressed and purified seven predicted OMPs of Brucella suis. The recombinant proteins were fused with 6-his and V5 epitope tags at their C termini to facilitate detection and purification. The B. suis surface genes were PCR synthesized based on their ORF sequences and directly cloned into an entry vector. The recombinant entry constructs were propagated in TOP 10 cells, recombined into a destination vector, pET-DEST42, then transformed into E. coli BL21 cells for IPTG-induced protein expression. The expressed recombinant proteins were confirmed with Western blot analysis using anti-6-his

15. SUBJECT TERMS
Brucella suis, outer membrane proteins, gene clone.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(Include area code)*
Unclass	Unclass	Unclass		8	

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Protein Expression and Purification 40 (2005) 134–141
Protein Expression & Purification
www.elsevier.com/locate/yprep

Cloning, expression, and purification of *Brucella suis* outer membrane proteins☆

Xuan Z. Ding[a,*], Apurba Bhattacharjee[a], Mikeljon P. Nikolich[a], Ian T. Paulsen[b], Gary Myers[b], Rekha Seshadri[b], David L. Hoover[a]

[a] *Division of CD&I, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC, USA*
[b] *The Institute for Genomic Research, MD, USA*

Received 13 September 2004, and in revised form 1 December 2004
Available online 6 January 2005

Abstract

Brucella, an aerobic, nonsporeforming, nonmotile Gram-negative coccobacillus, is a NIH/CDC category B bioterror threat agent that causes incapacitating human illness. Medical defense against the bioterror threat posed by *Brucella* would be strengthened by development of a human vaccine and improved diagnostic tests. Central to advancement of these goals is discovery of bacterial constituents that are immunogenic or antigenic for humans. Outer membrane proteins (OMPs) are particularly attractive for this purpose. In this study, we cloned, expressed, and purified seven predicted OMPs of *Brucella suis*. The recombinant proteins were fused with 6-His and V5 epitope tags at their C termini to facilitate detection and purification. The *B. suis* surface genes were PCR synthesized based on their ORF sequences and directly cloned into an entry vector. The recombinant entry constructs were propagated in TOP 10 cells, recombined into a destination vector, pET-DEST42, then transformed into *Escherichia coli* BL21 cells for IPTG-induced protein expression. The expressed recombinant proteins were confirmed with Western blot analysis using anti-6-His antibody conjugated with alkaline phosphatase. These *B. suis* OMPs were captured and purified using a HisGrab plate. The purified recombinant proteins were examined for their binding activity with antiserum. Serum derived from a rabbit immunized intramuscularly with dialyzed cell lysate of *Brucella* rough mutant WRR51. The OMPs were screened using the rabbit antiserum and purified IgG. The results suggested that recombinant *B. suis* OMPs were successfully cloned, expressed and purified. Some of the expressed OMPs showed high binding activity with immunized rabbit antiserum.

Keywords: *Brucella suis*; Recombinant membrane proteins; Antiserum; IFN-γ; Vaccine; Diagnostic reagents

Brucella is a zoonosis that is typically acquired after ingestion of foodstuffs, especially unpasteurized dairy products, or after occupational contact with infected animals. *Brucella* has been considered as an agent for biological warfare since the 1940s [1]. It is highly resistant to drying, easily survives aerosolization in water, and is highly infectious by multiple routes [2]. Human brucellosis is characterized by fever, chills, malaise, and a chronic course that requires six weeks of therapy with at least two antibiotics for cure treatment [3]. There are six recognized species of which *Brucella suis*, *Brucella melitensis*, and *Brucella abortus*, all smooth bacteria [4] that express long-chain *O*-polysaccharide (OPS) on their outer membrane lipopolysaccharide (LPS), are considered significant bioterror threat agents. All three agents are extremely similar at the DNA level.

To prevent *Brucella* infection, we developed a whole cell vaccine candidate, WR201. The whole cell vaccine has been tested in mice model and demonstrated that it was able to protect mice against intranasal challenge

☆ The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense. This work was supported by the Department of Defense, RAD IV.

* Corresponding author. Fax: +1 301 319 9123.
E-mail address: tom.ding@na.amedd.army.mil (X.Z. Ding).

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2004.12.017

with *B. melitensis* 16M [5]. These results indicate that some components of the bacteria function as immunogens, activate immune response, and induce a protective effect. Bacterial outer membranes are basically composed of protein, lipid, and sugar. It has been known that among the bacterial components, outer membrane proteins (OMPs) are particularly attractive used for development of vaccine candidates and diagnostic kits. For this reason, a few *Brucella* OMPs have been cloned, expressed, and identified from different species such as *B. melitensis* 20 kDa [6], 28 kDa [7], and 31 kDa [8] OMPs; *B. abortus* 16.5 kDa [9], 17 kDa [10], 22 kDa [11], 25 kDa [12], and 36 kDa [13] OMPs; and *B. ovis* 25 kDa OMP [14]. The molecular sizes of these OMPs are in the lower range and, they were constructed from a recombinant library and immune reaction as specific diagnostic agents has been examined.

Recently, the complete genomic sequences of *B. melitensis* [15] and *B. suis* [16] were published. The *B. suis* genome contains 3388 predicted open reading frames (ORFs), and versus 3197 ORFs for *B. melitensis*, on both chromosomes. Comparison of these sequences revealed extensive similarity and gene synteny [16]. Indeed, these two species differ by only 74 genes [17]. *B. suis* contains 42 unique genes found in 22 chromosomal regions and *B. melitensis* contains 32 unique genes in 11 locations [15–17]. Similarly, preliminary data from genome sequencing of *B. abortus* indicate that nearly all of this species' genes are identical to those of the other two. This genome information provides an opportunity to clone any expected gene and express its protein. In this study, we selected seven membrane proteins from *B. suis*, including three large ones of 88, 72, and 68 kDa, cloned them into Gateway system (Invitrogen), and expressed in *Escherichia coli*. Their immune reaction with antiserum derived from immunized rabbit was observed.

Materials and methods

Selection of Brucella OMP genes

Seven genes of *B. suis* that encoded different sizes of OMPs were selected and their bioinformation are shown in Table 1. Besides the seven genes, two controls were used for evaluation of gene expression. The negative control was pET-DEST-42 vector alone and the positive control was plasmid pET101/D/lacZ containing the lacZ gene.

Preparation of B.suis bacteria

One milliliter of *B. suis* bacteria was collected in a 50 ml tube and treated in an equal amount of phenol. After vortex, the bacterial cells were spun down at 1560*g*, for 10 min, at 4 °C. Nine hundred microliters of the upper liquid was collected into a 1.5 ml tube and added 630 μl isopropanol. The sample was centrifuged at 16,000*g*, for 10 min, at 4 °C. The pellet was washed with 70% alcohol and dissolved in 100 μl of sterile water.

PCR synthesis of B. suis genes

Primers used for synthesis of the seven *B. suis* genes were designed as 20 mers (Table 2). The forward primers contained an additional CACC sequence at the 5′ end followed by the first 20 bases of the ORF including the start codon. The reverse primer was comprised of the last 20 bases of the ORF prior to the stop codon. The PCR was conducted in a final volume of 50 μl that contained 5 μl of 10× ThermalAce reaction buffer, 1 μl of 50 mM dNTP, 1 μl of 1:10 diluted *B. suis* template, 0.5 μl of each 50 μM, 41 μl of PCR water, and 1 μl of ThermalAce. *B. suis* template without primers was used as negative control. The lacZ gene with primers from Invitrogen was used as a positive control. The PCR protocol was according to our previous study

Table 2
PCR primer sequences

No.	Primer sequence	
1	>5′-Cacc atgacggcaa gttctaaatt	<5′-Gaactttgtc gatacaccga
2	>5′-Cacc atggcgtttg agatttttgg	<5′-Aaacgcc tgtcctatgc
3	>5′-Cacc atgaattca ataggtctttt	<5′-Cgtatca ttttgtgtcc
4	>5′-Cacc gtgctcgcca gcacatctct	<5′-Gaacttg aaggccgtct gga
5	>5′-Cacc atggttgcgc ttggtatcgg	<5′-Cttctttgca gcgtcccggg
6	>5′-Cacc atggccgcaa ccgcagccgg	<5′-Cgtcttgcgc gcattcttga
7	>5′-Cacc atgaaacgct tccgcatcgt	<5′-Gccggcgttg cggcgggtga

Table 1
Genes and control plasmids used in demonstration expression experiment

No.	Name	Size (bp)	MW (Da)	AA
1	Outer membrane protein	2346	87,582	781
2	Outer membrane protein	1872	68,192	623
3	Outer membrane protein	1350	49,312	449
4	Outer membrane protein	1965	72,056	654
5	Outer membrane protein	753	27,289	250
6	Outer membrane protein	594	21,254	197
7	Outer membrane lipoprotein	381	13,259	126
–	Negative control	7400	0	0
+	Positive control	8825	121,000	

[18] designed as 1 cycle of 95 °C for 2 min; 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for $2\frac{1}{2}$ min; and 1 cycle of 72 °C for 10 min and then refrigeration at 4 °C.

Preparation of recombinant constructs in entry vector

The PCR products were directly cloned into an entry vector (Invitrogen). Reactions were performed according to the manufacturer's instructions. One microliter of PCR product plus 1 μl water was mixed with 0.5 μl of each salt solution and pENTR/SD/D-TOPO vector. Ligation was conducted at room temperature for 5 min. Three microliters of the ligation reaction was then added to 40 μl of TOP 10 competent cells and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added to 250 μl of culture medium and shaken at 37 °C for 1 h. After the incubation, 20 μl of TOP 10 cells was streaked on culture plate containing trypticase soy agar with 50 μg/ml kanamycin for clone selection. Plates were incubated at 37 °C overnight. Four clones of each gene were picked and cultured in 2 ml of LB broth medium with 50 μg/ml kanamycin for propagation of the plasmid. On the next day, the plasmids were purified using miniprep spin column 250 (Qiagen) and examined by PCR method.

Cloning of genes into destination vectors

One positive entry plasmid for each gene was selected for ligation into Gateway destination vector pET-DEST42. Each ligation reaction (LR) contained 2 μl of buffer, 4 μl of pENTR-gene, 2 μl of pET-DEST42 vector, and 2 μl of LR clonase. The reactions were incubated at 25 °C for 60 min. One microliter of proteinase K was added and the reaction mixture was incubated at 37 °C for another 10 min. One microliter of each LR was transformed into 50 μl of TOP 10 competent *E. coli* cells and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added to 250 μl of culture medium and shaken at 37 °C for 1 h. Twenty microliters of TOP 10 cells was then streaked on tissue culture plates containing trypticase soy agar with 100 μg/ml ampicillin for clone selection. Plates were incubated at 37 °C overnight. Four clones of each gene were picked and cultured in 2 ml of LB broth medium with 100 μg/ml ampicillin at 37 °C overnight. On the next day, the plasmids were purified and examined by above-mentioned methods.

Expression of recombinant proteins

Two microliters of the purified destination plasmids of each gene was mixed with 50 μl of expression host cells, BL21Star (DE3), and incubated at 4 °C for 30 min. After heating at 42 °C for 30 s, the cells were added with 250 μl of culture medium and shaken at 37 °C for 1 h. Then, the host cells were transferred into 5 ml of LB solution containing 100 μg/ml ampicillin and shaken at 37 °C overnight. On the next day, 200 μl of culture from each sample was transferred into 4 ml of LB medium containing 1% glucose and 100 μg/ml ampicillin, and shaken at 37 °C for another 2 h. The OD_{600} measurements of the cell cultures were within 0.5–0.8 U. Then, the culture was added IPTG (Invitrogen) to 1 μM and grown for 5 h. This optimal time was obtained based on preliminary experiments. After protein induction, cells were centrifuged and resuspended in 400 μl PBS for detection.

Western blot analysis of the expressed recombinant proteins

Seven microliters of protein sample for each gene was mixed with an equal amount of 2× SDS loading buffer. The samples were boiled for 10 min and purified on 13% acrylamide/bis gel. The protein samples were transferred onto nitrocellulose membrane and hybridized overnight with 1:2000 alkaline phosphatase (AP)-conjugated anti-6-His. The membranes were developed with 1 mg/ml of Naphthol AS-MX phosphate (Sigma) and 2 mg/ml of Fast red TR salt (Sigma) in 50 mM Tris buffer.

Purification and detection of recombinant proteins

A HisGrab plate (Pierce) was used to purify the recombinant proteins. One hundred microliters of IPTG-induced recombinant cells was centrifuged and the pellets were resuspended in 100 μl of lysis buffer. The samples were kept at room temperature for 30 min and then frozen and thawed three times. Ten microliters of the cell lysate was mixed with 90 μl of blotting buffer and placed on each well of the plate in triplicate, incubated at room temperature overnight. To detect the recombinant proteins, 100 μl of anti-V5 monoclonal antibody conjugated with AP (1:2000 dilutions) was added to each well and incubated at room temperature for 16 h. After washing three times with 1× PBS, 100 μl of developer buffer was added to each well and incubated at room temperature for 1 h. The protein concentration was determined using OD reader at wavelength 410 nm.

Antiserum screening of recombinant proteins

Rabbit serum was used for screening of the recombinant *Brucella* proteins. The rabbit was immunized intramuscularly with dialyzed cell lysate of *B. melitensis* rough mutant WRR51. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and

experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Two doses of vaccine (25 μg of protein per dose) were given 4 weeks apart. Blood was collected from the rabbit 2 weeks after the second dose of vaccine. IgG was prepared from the serum by affinity chromatography on protein G–Sepharose (Pharmacia-LKB Biotech). The IgG concentration in this preparation was 1.8 mg/ml. The immunized rabbit serum was diluted 1:4000 and added to each well of recombinant sample for 16 h of incubation. After washing six times with 1× PBS plus 0.5% Tween 20, the samples were incubated with 1:2000 secondary goat anti-rabbit antibody conjugated with HRP for 16 h. After substrate development, the plate was read with a plate reader.

Statistical analysis

All experiments were repeated at least three times. Data were processed with analysis of variance, Newman–Keuls, and Student's t test for comparison of groups. A value of $p < 0.05$ was used as the significance level for the study.

Results

Construction of B. suis outer membrane genes

Surface antigens of bacteria have been thought as the best immunogens to stimulate an immune response. Seven OMPs of *Brucella* with different molecular sizes were selected based on genome sequence of *B. suis* and their bioinformation has been described in Table 1. The OMP genes were PCR synthesized using ThermalAce DNA polymerase (Invitrogen). Genome DNA prepared from *B. suis* was used as template in the PCR. Result shows that single band with correct molecular weight has been amplified for each *Brucella* gene (Fig. 1).

To provide versatility for additional cloning for future applications, Gateway cloning system (Invitrogen) was selected to express the *Brucella* genes. The PCR produced *Brucella* genes were directly cloned into entry vector, pENTR directional TOPO vector (Invitrogen), and propagated in TOP 10 cells. The positive recombinant cells containing *Brucella* genes were selected on culture plate of LB agar with 50 μg/ml kanamycin. All seven samples resulted in isolated colonies on the selection plates. Four colonies of each gene were picked up and cultured in LB medium overnight for plasmid amplification. Plasmids were purified using QIApre column (QIAgen) and their molecular sizes of these were examined on 1% agarose gel (Fig. 2). Negative control was pENTR vector alone. The pENTR vector showed a lower molecular weight than the plasmids containing *Brucella* genes (data not shown). The presence of inserted genes in the plasmids was confirmed by PCR method, using primers designed according to the M13 sequence of the pENTR vector (data not shown). The full-length sequence of the gene inserts was further confirmed using ABI 3730xl capillary sequencing machines analysis by the Institute for Genomic Research. The results show that all the seven plasmids contain an insert that exactly matches OMP genes of *B. suis*.

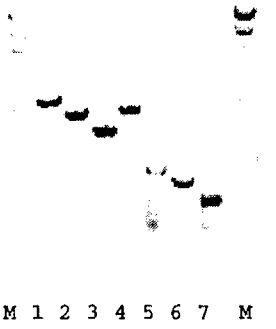

Fig. 1. PCR synthesis of OMP genes of *B. suis*. Seven OMP genes of *B. suis* were PCR synthesized using ThermalAce. The molecular weights of the genes have been described in Table 1. The PCR products were detected on 1% agarose gel. M represents λDNA/*Hin*dIII fragments (Invitrogen).

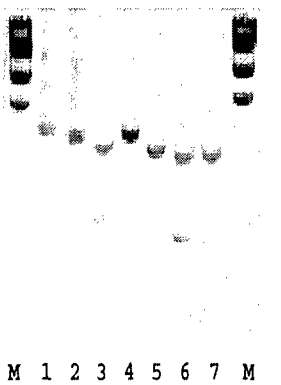

Fig. 2. pENTRY-*B. suis* plasmids examined on agarose gel. PCR products of *B. suis* OMP were cloned into pENTRY vector. The purified plasmids were examined on 1% agarose gel. M represents λDNA/*Hin*dIII fragments (Invitrogen).

One positive plasmid for each gene was selected for recombination into Gateway destination vector, pET-DEST42 (Invitrogen). The new constructs were propagated in TOP 10 cells. The positive recombinant cells were selected on culture plate of LB agar with 100 μg/ml ampicillin. Four clones of each gene were picked and cultured in LB medium with 100 μg/ml ampicillin at 37 °C overnight. Plasmids were purified from these samples and molecular sizes of these were examined on 1% agarose gel as described above. Insertion of *B. suis* genes in the destination constructs was confirmed by

PCR method using primers designed according to the attB sequence of the pET-DEST42 vector. Their sequences were attL1 5′-G TAC AAA AAA GCA GGC T-3′, and attL2 5′-GTA CAA GAA AGC TGG GT-3′. The PCR result shows that all seven constructs contain their recombinant inserts (Fig. 3).

Protein expression and measurement

Purified plasmids of each *B. suis* OMP were transformed into expression host cells, BL21Star (DE3) for protein expression. The positive control was cells transformed with pET-DEST42-lacZ and negative control was cells transformed with pET-DEST42 vector alone. The host cells were cultured in LB medium containing 1% glucose and 100 μg/ml ampicillin overnight. On the expression day, the recombinant cells were diluted in the ratio 1:20 into fresh LB medium containing 1% glucose and 100 μg/ml ampicillin, and cultured for another 2 h until OD_{600} measurements reached 0.5–0.8 U. Then, the cell culture of each sample was induced with 1 μM IPTG (Invitrogen). From preliminary experiments, the recombinant cells were shown to start expression of protein at 2 h and reached a maximum level at 4–5 h. Thus, optimal time for the IPTG-induced protein expression was 5 h after the chemical induction. After protein induction, cells were collected and resuspended in 1× PBS for detection.The samples were detected on Western blots using 1:2000 diluted monoclonal anti-6-His antibody (Invitrogen) to verify protein expression and their sizing. The protein samples were prepared with equal amount of 2× SDS loading buffer and purified on 13% acrylamide/bis gel as described under Materials and methods. The result shows that the seven OMPs of *B. suis* were over-expressed with correct molecular size in the host cells (Fig. 4).

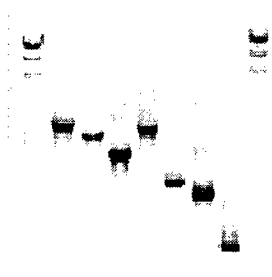

Fig. 3. PCR detection of pET-DEST42-*B. suis* OMP genes. These plasmids were detected with PCR method using primer of attB sequence of the pET-DEST42 vector. Five microliters of each PCR product was loaded on 1% agarose gel. M represents λDNA/*Hin*dIII fragments (Invitrogen).

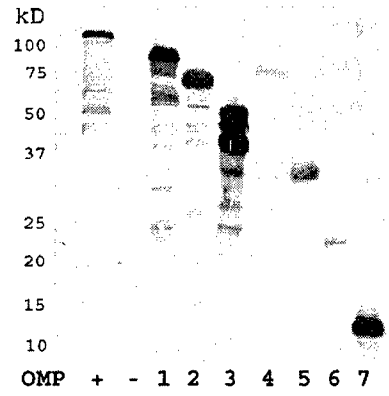

Fig. 4. Western blot analysis of expressed outer membrane proteins of *B. suis*. The protein samples were mixed with equal amount of 2× SDS loading buffer and boiled for 10 min. Fourteen microliters of each sample was loaded for each lane on 13% acrylamide/bis gel. The protein samples were transferred onto nitrocellulose membrane and hybridized with 1:2000 diluted monocloned antibody against 6-His. The membrane was developed with 1 mg/ml of Naphthol AS-MX phosphate (Sigma) and 2 mg/ml of Fast red TR salt (Sigma) in 50 mM of Tris buffer. + indicates pET-LacZ transformed cells and – indicates pET-DEST42 vector transformed cells. The precision plus protein standards provided by Bio-Rad were used as standard marker.

Purification of B. suis OMPs

It was necessary to purify and fix the recombinant proteins in a 96-well plate to detect their immune functions. HisGrab plate (Pierce) was used to purify the *Brucella* proteins in this study. Recombinant cells were treated with cell lysis buffer after the protein expression. HisGrab plate was pre-blotted with blotting buffer and then 10 μl cell sample plus 90 μl buffer were added into each well of the plate. The negative control was *Brucella* cell lysate and the positive was LacZ recombinant protein. Protein samples were incubated at room temperature overnight. After wash, 100 μl of anti-V5 monoclonal antibody conjugated with AP (1:2000) was added to each well and incubated with the sample at room temperature overnight. The bound proteins were detected using phosphatase substrate. The result shows that LacZ protein was recognized by the antibody, but not *Brucella* lysate. All seven *Brucella* recombinant proteins were positively identified by the anti-V5 antibody (Fig. 5).

Screening Brucella proteins for immune reactivity against rabbit serum

We have found that intranasal immunization of rhesus macaques with WR201 leads to production of anti-*Brucella* antibody and protects rhesus macaques from infectious brucellosis induced by subsequent aerosol challenge with 16M [5]. To investigate the possibility that the recombinant *Brucella* proteins react with protective antiserum, serum was collected from an immunized rabbit and used as a tool to screen for expressed

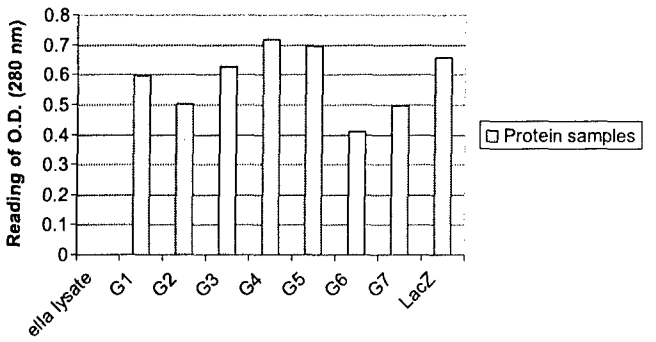

Fig. 5. Purification and identification of recombinant *B. suis* OMPs. Seven OMPs of *B. suis* were purified using HisGrab plate and detected using anti-V5 monoclonal antibody. *Brucella* cell lysate was used as negative control and pET-lacZ transformed cells were used as positive control.

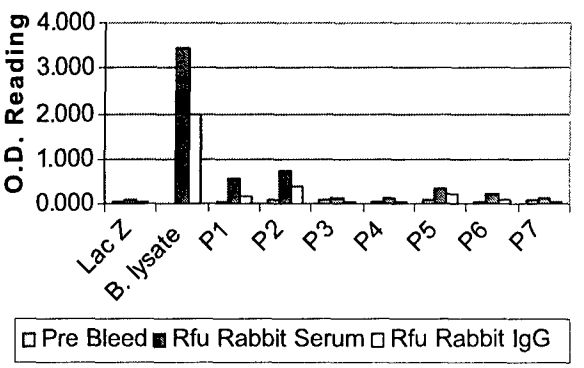

Fig. 6. Screening of recombinant proteins using immunized rabbit serum and IgG. *Brucella* recombinant OMPs were purified with HisGrab plate and then incubated with 1:4000 diluted normal or immunized rabbit serum or IgG. Secondary antibody was goat anti-rabbit with 1:2000 dilution. The negative control was recombinant LacZ protein and the positive control was *Brucella* cell lysate.

outer membrane proteins of *Brucella*. Two controls were used in the experiment. The positive control was a rough *Brucella* bacterial lysate and the negative control was expressed LacZ recombinant protein. The recombinant *Brucella* proteins were precipitated using a His-Grab plate overnight. Unbound proteins were washed out after the incubation. The purified recombinant proteins were then reacted with 1:4000 diluted rabbit serum. Fig. 6 shows that *Brucella* cell lysate reacted strongly with immunized rabbit serum and isolated IgG, but LacZ protein had no reaction with them. Normal rabbit serum had no reaction with any of the samples. Recombinant proteins 1 and 2 reacted significantly with the immunized rabbit serum. This result indicates that certain surface antigens may directly cause the production of protective antiserum.

Discussion

Development of a human vaccine and improved diagnostic tests would significantly assist prevention of *Brucella*-induced infection and serve a valuable societal goal. A *Brucella* whole cell vaccine, WR201, has been developed and found that it was attenuated and able to protect wild type virulent *B. melitensis* induced infection in both mice and monkey models [5]. Based on these data, the discovery of additional immunogenic or antigenic bacterial constituents is a vital component of the vaccine and diagnostics development process. Outer membrane proteins of the bacteria have been reported to play an important role during *Brucella* infection and induction of host immune response. Generally, successful vaccines and diagnostic reagents against their microorganisms primarily induce antibodies against surface structures. These surface proteins of the bacteria have been thought of as useful antigens for development of both diagnostic reagents and vaccine candidates. Some *Brucella* proteins have been cloned and expressed, but they were constructed by traditional methods from an unknown gene library as genome information of the bacteria was not available in those days. Full-length genome sequences of *B. suis* and *B. melitensis* have been published in 2002 [16], and this bioinformation provides an extremely useful opportunity to clone and express the *Brucella* outer membrane proteins. The utility of applying whole-genome sequencing data to development of recombinant proteins has been demonstrated for *Neisseria meningitidis* [19] and *Streptococcus pneumoniae* [20]. In this study, we constructed and expressed seven OMPs of *B. suis* based on open reading frames of the genome information.

The full-length sequence of the seven ORFs was PCR synthesized and cloned into the Gateway expression system. The system provides a benefit for optimal expression of these *Brucella* membrane proteins using alternative destination vectors for different purposes. These expressed recombinant proteins have been designed to be fused with both V5 and 6-His tags at their C termini. Thus, two alternative methods can be used to purify and detect them, either to capture V5 or 6-His tags and then either to detect 6-His or V5 protein tags. The two methods were compared and we found that the recombinant proteins can be captured or detected by using either of the methods (Fig. 7).

Since nickel reagent may be used to capture those proteins fused with 6-His tag, His-Grab plate (Qiagen) was used in the experiment to purify these expressed recombinant proteins. In this study, all expressed proteins were confirmed by Western blot and then purified using the His-Grab plate. High protein purity is required, as the recombinant proteins will be further examined, for their antigenic functions. In another study, we have analyzed four expressed recombinant proteins before and after purification with a Ni–NTA spin column (Pierce) following the manufacturer's instructions. The purification principle of the column is the same as the HisGrab plate, but provides sufficient protein to visualize by gel

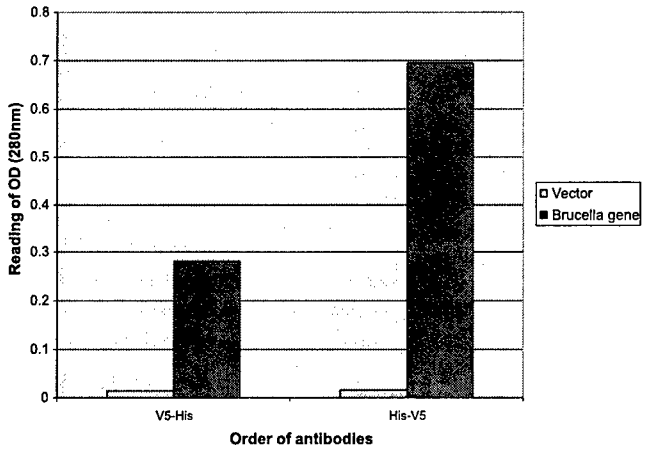

Fig. 7. Order of capture and detection using different antibodies. V5-His indicates that antibody against V5 was used to capture and anti-6-His antibody was used to detect the recombinant proteins. His-V5 indicates that antibody against 6-His was used to capture and anti-V5 antibody was used to detect the recombinant proteins.

electrophoresis. After elution, the purified proteins were examined using both Coomassie blue stain and Western blot. Only a single band was detected from each of the purified protein samples, while the cell lysates contained multiple bands (data not shown). These results indicate that the expressed recombinant proteins are well purified by using a nickel-based method, suggesting that this method should be adequate for analysis of antigenicity.

It is very possible that certain protein components of the *Brucella*, but not necessary as opposed to whole cell, may be sufficient to elicit the protective immunity with reduced side effects. These bacterial proteins can be cloned and expressed by using our currently used method and are examined for their antigenic activity. Immunized animals that survived from virulent bacterial challenge may have actively obtained protective immunities. The protective antibodies or/and T lymphocytes derived from these hosts should recognize and react with those antigen proteins that cause the infection and produce the specific protective immunities. Thus, these antibodies and activated T lymphocytes can be used as screening probes to select out those proteins that are immunogenic. Antiserum was collected from the rabbit immunized intramuscularly with dialyzed cell lysate of *B. melitensis* rough mutant WRR51. The immunized rabbit serum and IgG were diluted 1:4000, and used to screen the recombinant surface antigens. Both the antiserum and derived IgG had strong reaction to the *Brucella* cell lysate. In fact, we found that using whole cell lysate of WRR51 as antigen we get more than 20 protein bands in Western blot using this antiserum (data not shown). Recombinant OMPs 1 and 2 were also found to have significant detection by the antiserum. The protein samples were prepared by treatment of WRR51 cells and the recombinant proteins with sodium dodecyl sulphate (SDS), therefore, the proteins were in their denatured form. The antibodies generated in rabbit will therefore recognize linear epitopes of the proteins.

Intraperitoneal administration of WR201 to mice leads to not only production of antibody to OMPs, but also induction of spleen cells that produce IFN-γ when cultured with *Brucella* antigens, and protection against intranasal challenge with 16M [5]. This result indicates that both humoral and cellular immunities may be important in the protection of host against *Brucella* infection. In a pilot experiment, we observed the effect of the immobilized recombinant surface antigens on IFN-γ production of spleen cells derived from mice immunized orally with 10^{11} colony-forming units (CFU) of WR201 for 8 weeks and found that at least two of the recombinant proteins significantly increased IFN-γ (data not shown). These results indicate that surface antigens may play a distinct role in stimulation of host immune system. These recombinant surface antigens will be further examined in vivo model to test their immune stimulation.

Acknowledgments

Authors thank Mr. P. Adams for preparing ELISA experiments and Ms. L. Young for preparing Western blot analysis. This work was supported by DOD RAD IV.

References

[1] Army, D. o. t., U.S. Army Activity in the United States Biological Warfare Programs, vols 1 and 2, 24 February 1977 (Unclassified).
[2] S.S. Elberg, D.W. Henderson, Respiratory pathogenicity of *Brucella*, J. Infect Dis. 82 (1948) 302–306.
[3] E.J. Young, An overview of human brucellosis, Clin. Infect Dis. 21 (1995) 283–289.
[4] E. Moreno, D. Borowiak, H. Mayer, *Brucella* lipopolysaccharides and polysaccharides, Ann. Inst. Pasteur. Microbiol. 138 (1987) 102–105.
[5] D.L. Hoover, R.M. Crawford, L.L. VanDeVerg, M.J. Izadjoo, A.K. Bhattacharjee, C.M. Paranavitana, R.L. Warren, M.P. Nikolich, T.L. Hadfield, Protection of mice against brucellosis by vaccination with *Brucella melitensis* WR201(16MDeltapurEK), Infect. Immun. 67 (1999) 5877–5884.
[6] P.A. Denoel, M.S. Zygmunt, V. Weynants, A. Tibor, B. Lichtfouse, P. Briffeuil, J.N. Limet, J.J. Letesson, Cloning and sequencing of the bacterioferritin gene of *Brucella melitensis* 16M strain, FEBS Lett. 361 (2-3) (1995) 238–242.
[7] L.E. Lindler, T.L. Hadfield, B.D. Tall, N.J. Snellings, F.A. Rubin, L.L. VanDeVerg, D.L. Hoover, R.L. Warren, Cloning of a *Brucella melitensis* group 3 antigen gene encoding Omp28, a protein recognized by the humoral immune response during human brucellosis, Infect. Immun. 64 (1996) 2490–2499.
[8] N. Vizcaino, A. Cloeckaert, M.S. Zygmunt, G. Dubray, Cloning, nucleotide sequence, and expression of the *Brucella melitensis* omp31 gene coding for an immunogenic major outer membrane protein, Infect. Immun. 64 (1996) 3744–3751.

[9] A. Tibor, V. Weynants, P. Denoel, B. Lichtfouse, X. De Bolle, E. Saman, J.N. Limet, L.J.J. Letesson, Molecular cloning, nucleotide sequence, and occurrence of a 16.5-kDa outer membrane protein of *Brucella abortus* with similarity to pal lipoproteins, Infect. Immun. 62 (1994) 3633–3639.

[10] F. Hemmen, V. Weynants, T. Scarcez, J.J. Letesson, E. Saman, Cloning and sequence analysis of a newly identified *Brucella abortus* gene and serological evaluation of the17-kDa antigen that it encodes, Clin. Diagn. Lab. Immunol. 2 (1995) 263–267.

[11] A. Tibor, E. Saman, P. de Wergifosse, A. Cloeckaert, J.N. Limet, J.J. Letesson, Molecular characterization, occurrence, and immunogenicity in infected sheep and cattle of two minor outer membrane proteins of *Brucella abortus*, Infect. Immun. 64 (1996) 100–107.

[12] P. de Wergifosse, P. Lintermans, J.N. Limet, A. Cloeckaert, Cloning and nucleotide sequence of the gene coding for the major 25-kDa outer membrane protein of *Brucella abortus*, J. Bacteriol. 177 (1995) 1911–1914.

[13] T.A. Ficht, S.W. Bearden, B.A. Sowa, L.G. Adams, DNA sequence and expression of the 36-kDa outer membrane protein gene of *Brucella abortus*, Infect. Immun. 57 (1989) 3281–32891.

[14] A. Cloeckaert, J.M. Verger, M. Grayon, M.S. Zygmunt, O. Grepinet, Nucleotide sequence and expression of the gene encoding the major 25-kDa outer membrane protein of *Brucella ovis*: evidence for antigenic shift, compared with other *Brucella* species, due to a deletion in the gene, Infect. Immun. 64 (1996) 2047–2055.

[15] V.G. DelVecchio, V. Kapatral, R.J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P.H. Elzer, S. Hagius, D. O'Callaghan, J.J.R. Haselkorn, N. Kyrpides, R. Overbeek, The genome sequence of the facultative intracellular pathogen *Brucella melitensis*, Proc. Natl. Acad. Sci. USA 99 (2002) 443–448.

[16] I.T. Paulsen, R. Seshadri, K.E. Nelson, J.A. Eisen, J.F. Heidelberg, T.D. Read, R.J. Dodson, L. Umayam, L.M. Brinkac, M.J. Beanan, S.C. Daugherty, R.T. Deboy, A.S. Durkin, J.F. Kolonay, R. Madupu, W.C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S.E. Van Aken, S. Riedmuller, H. Tettelin, S.R. Gill, O. White, S.L. Salzberg, D.L. Hoover, L.E. Lindler, S.M. Halling, S.M. Boyle, C.M. Fraser, The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts, Proc. Natl. Acad. Sci. USA 99 (2002) 13148–13153.

[17] R.M. Tsolis, Comparative genome analysis of the alpha-proteobacteria: relationships between plant and animal pathogens and host specificity, Proc. Natl. Acad. Sci. USA 99 (2002) 12503–12505.

[18] X.Z. Ding, R.C. Smallridge, R.J. Galloway, J.G. Kiang, Rapid assay of HSF1 and HSF2 gene expression by RT-PCR, Mol. Cell. Biochem. 158 (1996) 189–192.

[19] M. Pizza, V. Scarlato, V. Masignani, M.M. Giuliani, B. Arico, M. Comanducci, G.T. Jennings, L. Baldi, E. Bartolini, B. Capecchi, C.L. Galeotti, E. Luzzi, R. Manetti, E. Marchetti, M. Mora, S. Nuti, G. Ratti, L. Santini, S. Savino, M. Scarselli, E. Storni, P. Zuo, M. Broeker, E. Hundt, B. Knapp, E. Blair, T. Mason, H. Tettelin, D.W. Hood, A.C. Jeffries, N.J. Saunders, D.M. Granoff, J.C. Venter, E.R. Moxon, G. Grandi, R. Rappuoli, Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing, Science 287 (2000) 1816–1820.

[20] T.M. Wizemann, J.H. Heinrichs, J.E. Adamou, A.L. Erwin, C. Kunsch, G.H. Choi, S.C. Barash, C.A. Rosen, H.R. Masure, E. Tuomanen, A. Gayle, Y.A. Brewah, W. Walsh, P. Barren, R. Lathigra, M. Hanson, S. Langermann, S. Johnson, S. Koenig, Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection, Infect. Immun. 69 (2001) 1593–1598.